FORM PTO-1390 REV. 2/01T — U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 03495 0213

U S APPLICATION NO (If known, see 37 CFR 1.5): 09/980839

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/01620	June 9, 2000	June 9, 1999

TITLE OF INVENTION
EARLY DETECTION OF FLAVIVIRUSES USING THE NS1 GLYCOPROTEIN

APPLICANT(S) FOR DO/EO/US
Marie FLAMAND, Françoise MEGRET, Sophie ALCON, Antoine TALARMIN, Philippe DESPRES, and Vincent DEUBEL

Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☐ is attached hereto (required only if not communicated by the International Bureau.
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed with the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☒ is attached hereto
   b. ☐ has been previously submitted under 35 U S.C. 154 (d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C 371 (c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A Substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154 (d)(4).
19. ☐ A second copy of the English language translation of the international application 35 U.S.C 154 (d)(4).
20. ☒ Other items or information:
   a. ☒ Copy of cover page of International Publication No. WO 00/75665
   b. ☒ Sequence Listing (3 sheets)
   c. ☐

09980839 .060102

JC10 Rec'd PCT/PTO 07 DEC 2001

U.S. APPLICATION NO (If known, see 37CFR 1 5) 09/980839	INTERNATIONAL APPLICATION NO. PCT/FR00/01620	ATTORNEY'S DOCKET NUMBER 03495 0213

21. ☒ The following fees are submitted.

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTOand all claims satisfied provisions of PCT Article 33 (1)-(4) **$100.00**

			ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				$	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	20	- 20 =		x $18 00	$	
Independent Claims	8	-3 =	5	x $84.00	$420.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+$280 00	$	
			TOTAL OF THE ABOVE CALCULATIONS =		$1310.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.					$	
			SUBTOTAL =		$1310.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest priority date (37 CFR 1.492(f)).					$	
			TOTAL NATIONAL FEE =		1310.00	
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +					$	
			TOTAL FEES ENCLOSED =		$1310.00	
					Amount to be refunded:	$
					charged:	$

a. ☒ A check in the amount of $ 1310.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-0916. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.
1300 I Street, N.W.
Washington, D.C. 20005-3315

DATED: December 7, 2001

SIGNATURE

Ernest F. Chapman/25,961

NAME/REGISTRATION NO.

PATENT
Customer No. 22,852
Attorney Docket No. 03495-0213

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
FLAMAND et al.	Group Art Unit: Not yet assigned
Application No.: 09/980,839	Examiner: Not yet assigned
Filed: December 7, 2001	
For: EARLY DETECTION OF FLAVIVIRUS USING THE NS1 GLYCOPROTEIN	

Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to the examination of the above application, please amend this application as follows:

IN THE SPECIFICATION:

Please insert the following paragraph after the title on page 1, line 2 of the specification:

This application is based on and claims the benefit of French applications 99/07290, filed June 9, 1999, and 99/07361, filed June 10, 1999, and International application PCT/FR00/01620, filed June 9, 2000. The entire disclosure of these applications are relied upon and incorporated by reference herein.

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Please amend the second paragraph on page 16, lines 21-24, with the following paragraph:

Figure 2 represents the sequence of the NS1 protein of dengue virus serotype 1 (SEQ ID NO: 1), obtained with clone 4C of Example 2 below, and also the corresponding coding sequence (SEQ ID NO: 2).

Please replace the Sequence Listing originally filed with the application with the Sequence Listing submitted herewith.

IN THE CLAIMS:

Please cancel claims 1-20.

Please add the following new claims:

21. (NEW) A method for the early detection of a flaviviral infection comprising: detecting an NS1 nonstructural glycoprotein of a flavivirus in a biological sample by an immunological method using at least two antibodies,

wherein the biological sample is obtained at anytime throughout the duration of the clinical phase of the infection,

wherein the two antibodies may be identical or different,

wherein a first antibody, called a capture antibody, is either (1) a polyclonal antibody preselected by immunocapture on the NS1 protein of the flavivirus, wherein the NS1 protein is in hexameric form, or (2) a mixture of purified anti-NS1 monoclonal antibodies preselected for their high affinity for the NS1 protein of the flavivirus, wherein the NS1 protein is in hexameric form, and

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wherein a second antibody, called a revelation antibody, is a polyclonal antibody directed against the NS1 protein in hexameric form or a mixture of monoclonal antibodies directed against an NS1 protein in hexameric form.

22. (NEW) The method as claimed in claim 21, wherein the flaviviral infection is an infection of the dengue virus.

23. (NEW) The method as claimed in claim 21, wherein the first antibody is attached to a solid support and the second antibody is optionally conjugated to a label.

24. (NEW) The method as claimed in claim 23, wherein if the second antibody is not conjugated to a label, binding of the second antibody to the NS1 protein attached to the solid support is detected with a third antibody conjugated to a label.

25. (NEW) The detection method as claimed in claim 24, wherein the label conjugated to the third antibody is an enzyme.

26. (NEW) The detection method as claimed in claim 25, wherein

(A) the first antibody is a mouse polyclonal antibody selected by immunocapture of the NS1 protein of the dengue virus, wherein the NS1 protein is in hexameric form, and

(B) the second antibody is a polyclonal antibody from a rabbit immunized with the NS1 protein of dengue virus serotype 1, wherein the NS1 protein is in hexameric form,

(C) the third antibody reveals binding of the second antibody to NSI protein, and the third antibody is an antibody conjugated to peroxidase and directed against the second antibody.

27. (NEW) A boxed set for the early diagnosis of a flaviviral infection, comprising:

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(A) a first antibody, called a capture antibody, which is either (1) a polyclonal antibody preselected by immunocapture on the NS1 protein of the flavivirus, wherein the NS1 protein is in hexameric form or (2) a mixture of purified anti-NS1 monoclonal antibodies preselected for their high affinity for the NS1 protein of the flavivirus, wherein the NS1 protein is in hexameric form;

(B) a second antibody, called a revelation antibody, is a polyclonal antibody directed against NS1 protein in hexameric form or a mixture of monoclonal antibodies directed against a NS1 protein in hexameric form;

(C) at least one positive control comprising an NS1 protein of a flavivirus, wherein the NS1 protein is in hexameric form; and,

(D) at least one negative control comprising a normal, uninfected human serum.

28. (NEW) The boxed set as claimed in claim 27, wherein the NS1 protein is obtained from a culture supernatant either from infected mammalian cells or from mammalian cells transfected with a recombinant plasmid comprising a gene for an NS1 protein of a flavivirus or a fragment of the gene or a fragment of the flaviviral genome, the fragments being capable of expressing all or part of the NS1 protein.

29. (NEW) The boxed set as claimed in claim 27 wherein the NS1 protein is from a dengue virus.

30. (NEW) The boxed set for the early diagnosis of a flaviviral infection as claimed in claim 28, wherein the recombinant plasmid was deposited with the Collection Nationale de Cultures et de Microorganismes under the number I-2220.

31. (NEW) A method for purifying an NS1 protein of a flavivirus, wherein the NS1 protein is in hexameric form, from a culture supernatant either of infected

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(A) a first antibody, called a capture antibody, which is either (1) a polyclonal antibody preselected by immunocapture on the NS1 protein of the flavivirus, wherein the NS1 protein is in hexameric form or (2) a mixture of purified anti-NS1 monoclonal antibodies preselected for their high affinity for the NS1 protein of the flavivirus, wherein the NS1 protein is in hexameric form;

(B) a second antibody, called a revelation antibody, is a polyclonal antibody directed against NS1 protein in hexameric form or a mixture of monoclonal antibodies directed against a NS1 protein in hexameric form;

(C) at least one positive control comprising an NS1 protein of a flavivirus, wherein the NS1 protein is in hexameric form; and,

(D) at least one negative control comprising a normal, uninfected human serum.

28. (NEW) The boxed set as claimed in claim 27, wherein the NS1 protein is obtained from a culture supernatant either from infected mammalian cells or from mammalian cells transfected with a recombinant plasmid comprising a gene for an NS1 protein of a flavivirus or a fragment of the gene or a fragment of the flaviviral genome, the fragments being capable of expressing all or part of the NS1 protein.

29. (NEW) The boxed set as claimed in claim 27 wherein the NS1 protein is from a dengue virus.

30. (NEW) The boxed set for the early diagnosis of a flaviviral infection as claimed in claim 28, wherein the recombinant plasmid was deposited with the Collection Nationale de Cultures et de Microorganismes under the number I-2220.

31. (NEW) A method for purifying an NS1 protein of a flavivirus, wherein the NS1 protein is in hexameric form, from a culture supernatant either of infected

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mammalian cells or of mammalian cells transfected with a recombined plasmid, comprising:

(A) expressing the NSI protein or a fragment of the NS1 protein from an NS1 gene or a fragment of the flaviviral genome, wherein the fragments are capable of expressing the NS1 protein prior to the purification of the NS1 protein;

(B) treating the NSI protein with a precipitating agent;

(C) centrifuging the treated NSI protein; and,

(D) separating a soluble form of the NS1 protein from a microparticulate form of NS1 protein.

32. (NEW) The method for purifying NS1 protein as claimed in claim 31, wherein the flavivirus is a dengue virus.

33. (NEW) The method for purifying NS1 protein as claimed in claim 32, wherein the flavivirus is dengue virus serotype 1.

34. (NEW) An immunogenic composition, comprising as the active principle, an NS1 protein of a flavivirus, wherein the NS1 protein is in hexameric form, optionally associated with other proteins, and at least one pharmaceutical vehicle.

35. (NEW) The immunogenic composition as claimed in claim 34, wherein the composition further comprises at least one mixture of NS1 proteins in hexameric form of a dengue virus serotype.

36. (NEW) A method for preparing an immunogenic composition capable of inducing the production of antibodies *in vivo* comprising combining an NS1 protein in hexameric form, or an NS1 protein expressed from a system for the expression of NS1 protein in hexameric form, with at least one pharmaceutical vehicle.

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37. (NEW) A method for manufacturing a medicinal product capable of inducing passive immunization comprising producing at least one monoclonal anti-NS1 antibody having a high affinity for NS1 protein in hexameric form, purifying the monoclonal antibody, and modifying the monoclonal antibody by selecting for Fab fragments or humanizing the monoclonal antibody, wherein the hexameric form is nondegraded.

38. (NEW) A method for selecting, *in vitro*, specific anti-NS1 antibodies able to diagnose an infection with a flavivirus, at an early stage, comprising binding the antibodies to NS1 protein in hexameric form, the hexameric form being nondegraded.

39. (NEW) An immunogenic composition, comprising an active principle and a pharmaceutical vehicle, wherein the active principle is either a polynucleotide capable of expressing all or part of an NS1 protein of a dengue virus of any serotype, or the active principle is an expression system comprising at least one promoter capable of expressing, in a host into which it is injected, a DNA encoding an NS1 protein of a dengue virus of any serotype.

40. (NEW) A method for expressing a polynucleotide encoding an NS1 protein of a dengue virus, comprising associating a polynucleotide of SEQ ID No. 1 with a promoter for said polynucleotide, and expressing the polynucleotide in a eukaryotic cell.

REMARKS

Entry of this Preliminary Amendment is respectfully requested.

The amendment to the specification adds sequence identifiers, and therefore does not add new matter. The substitute Sequence Listing is submitted in accordance with 37 C.F.R. § 1.825 and does not add new matter. A computer readable form of the

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Sequence Listing is also submitted herewith, and contains the same information as the substitute Sequence Listing.

New claims 21-40 are derived from the subject matter of original claims 1-20. The new claims were drafted to conform to United States patent practice. These new claims do not add new matter.

If there is any fee due in connection with the filing of this Preliminary Amendment, please charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Dated: June 21, 2002

By: _____

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Appendix to the Preliminary Amendment of June 21, 2002

IN THE SPECIFICATION

Please amend the second paragraph on page 16, lines 21-24, with the following paragraph:

Figure 2 represents the sequence of the NS1 protein of dengue virus serotype 1 (SEQ ID NO: 1), obtained with clone 4C of Example 2 below, and also the corresponding coding sequence (SEQ ID NO: 2).

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8/PRTS

EARLY DETECTION OF FLAVIVIRUSES USING THE NS1 GLYCOPROTEIN

The present invention relates to a method for the early detection of flaviviruses, in particular of the dengue virus, and to the application thereof.

Dengue is an acute febrile tropical disease and the virus which causes it is an arbovirus which is transmitted by mosquitoes. The vectors of the disease are mosquitoes of the *Aedes* genus, in particular *Aedes aegypti*, which most commonly leave their larvae in domestic and peridomestic areas. The responsible virus, isolated in 1951, has been classified into four different antigenic types (DEN1, DEN2, DEN3 and DEN4). It belongs to the *Flaviviridae* family, genus flavivirus.

More than two billion inhabitants live in endemic regions and the number of individuals infected by the virus is thought to be more than 100 million per year. Dengue is in particular responsible for 500 000 hospitalizations and for several tens of thousands of deaths annually, mostly children.

After an incubation of five to eight days, the clinical signs generally begin suddenly and consist of the appearance of undifferentiated fever (DF *dengue fever*) accompanied by severe headaches, lumbago, muscle and joint pain and also shivering. From the third to the fifth day of the febrile phase, a congestive maculopapular rash may appear for three to four days (conventional dengue).

In its severe form, the infection may result in the appearance of a hemorrhagic syndrome (DHF or *dengue hemorrhagic fever*), characterized by increased vascular permeability and deregulation of hemostasis. Although,

in the majority of cases, the disease generally evolves favorably within a week, it may turn out to be fatal in the event of hypovolemic shock (*DSS* or *dengue shock syndrome*). These complications may be due to the presence of preexisting immunity, acquired in particular during a primary infection with a heterologous dengue virus (different serotype). Specifically, two different types of serological response are identified in individuals infected with dengue: individuals who have never suffered a flavivirus infection and have not been vaccinated against another flavivirus (yellow fever virus, Japanese encephalitis virus for example) will exhibit a primary response, characterized by a slow appearance of antibodies specific for the virus responsible for the infection; individuals who have already suffered a flavivirus infection (other dengue serotype for example) or have been vaccinated against another flavivirus will exhibit a secondary response, characterized by the rapid appearance of antibodies.

The infectious agent is the dengue virus which belongs to the *Flaviviridae* family, to which the yellow fever virus and the Japanese encephalitis virus also belong (T.P. Monath et al., (1996) Flaviviruses in B.N. Fields, D.M. Knipe, P.M. Howly et al. (eds.) "Fields Virology" Philadelphia: Lippincott Raven Press Publishers). These viruses have a single-strand RNA with positive polarity which comprises 11 000 nucleotides and which encodes a polyprotein of approximately 3400 amino acids. It is separated into three structural proteins and seven nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, during co-translational and post-translational cleavage by viral and cellular proteases. The NS1 nonstructural protein was identified for the first time in 1970 by P.K. Russel et al. (*J. Immunol.*, (1970), **105**, 838-845) and characterized in 1985 by G.W. Smith et al. (*J. Gen Virol.*, (1985), **66**, 559-571). This glycoprotein, which

is highly conserved in the flavivirus genus (T.P. Monath already mentioned), in particular in the four dengue virus serotypes, exists in an intracellular form and in an extracellular form. The intracellular form is thought to be involved in the early phases of replication of the virus (Hall R.A. et al., *J. Virol.* (1999), **73**, 10272-10280; Rice C.M. et al., *J. Virol.*, (1997), **71**, 291-298; Rice C.M. et al., *J. Virol.*, (1996), **222**, 159-168; Rice C.M. et al., *J. Virol.*, (1997), **71**, 9608-9617). Before being transported to the plasma membrane, the NS1 protein undergoes dimerization. In mammalian cells, but not in insect cells, a portion of the NS1 protein is released into the extracellular medium, either primarily in the form of a soluble protein, or secondarily in a microparticulate form. When it is in a soluble form, the protein exists in the form of an oligomer, in particular of a pentamer or of a hexamer (Crooks A.J. *et al. J. Chrom.* (1990), **502**, 59-68 and *J. Gen. Virol.* (1994), **75**, 3453-3460). At the current time, the biological function of the NS1 protein is unknown.

Several studies suggest that the NS1 protein is immunodominant in nature in the protective immune response against flavivirus infections. Experiments carried out with a certain number of flaviviruses, such as the yellow fever, dengue, Japanese encephalitis and tick-borne encephalitis viruses, have shown partial or total protection against a lethal dose of homologous virus in animals vaccinated using the subunit NS1 protein or the NS1 protein produced by virus vectors, of the vaccinia or adenovirus type (Schlesinger *et al.*, J. Virol (1986), **60**, 1153-1155; *J. Gen. Virol.*, (1987), **68**, 853-857; Falgout *et al. J. Virol.*, (1990), **64**, 4356-4363; Jacobs *et al. J. Virol.*, (1992), **66**, 2086-2095; Hall *et al. J. Gen. Virol.*, (1996), **77**, 1287-1294; Konishi *et al., Virology*, (1991), **185**, 401-410).

Passive immunization of mice with monoclonal anti-NS1 antibodies has also made it possible to obtain a certain degree of protection (Schlesinger *et al., J. Immunol.* (1985), **135**, 2805-2809; Gould *et al. J. Gen. Virol.*, (1986), **67**, 591-595; Henchal *et al., J. Gen. Virol.*, (1988), **69**, 2101-2107). The role of anti-NS1 antibodies in the protection is not entirely known. It may be that the NS1 proteins at the surface of infected cells are recognized by complement-fixing antibodies, leading to lysis of the infected cells (Schlesinger *et al., Virology*, (1993), **192**, 132-141).

No specific treatment exists and the care given to the patient is uniquely symptomatic. In the case of conventional dengue, the treatment is based on the administration of analgesics and antipyretics. In the case of DHF, the treatment consists of an infusion to compensate for the plasma leakage, combined with correction of hydroelectric problems and reinitiation of diuresis.

There is no commercially available vaccine against the dengue virus. On the other hand, protection assays with attenuated strains of the 4 dengue virus serotypes have been carried out by N. Bhamarapravati *et al.* (*Dengue and Dengue haemorrhagic fever* (1997), 367-377), with unsatisfactory results. Prevention is therefore based solely on combating the vector. This combat combines larval destruction and "adulticide" spraying.

In the absence of a vaccine, it is necessary to monitor epidemics and to prevent the abovementioned complications; to do this, active monitoring programs have in particular been set up by the World Health Organization, and essentially comprise the monitoring of cases of fever and of vector insects, and the serological and virological screening of individuals having a fever and suspected of being infected with the dengue virus.

The etiology of dengue is sometimes tricky to affirm when a patient exhibits a dengue-like undifferentiated febrile syndrome, the cause of which may be another arbovirus, viruses which cause eruptive fevers such as the flu, or nonviral pathogens which are agents of diseases such as leptospirosis and even malaria. Only a laboratory test can provide the diagnosis.

At the current time, several tests exist for diagnosing dengue. However, in order to obtain an interpretable result, it is necessary to combine several methods:

- isolation of the virus, by conventional virology techniques, in particular by infection of cell cultures or propagation in the brain of young mice or amplification by inoculation into mosquitoes, and examination, for example, by immunofluorescence. These methods have the drawback of being difficult to carry out and of depending on the sample being taken early and on good conditions of conservation; in addition, the first results cannot be obtained in less than a week; in order to overcome these drawbacks, use may be made of an RT/PCR test (V. Deubel, *L'eurobiologiste* (1997), volume XXXI, 37-155); however, this means is not always reliable and cannot be used routinely in the countries to which the dengue virus relates, for reasons of cost and equipment;

- serological tests; the earliest serological diagnosis consists in searching for IgMs specific for viral antigens using the MAC-ELISA (*immunoglobulin M Antibody Capture Enzyme-Linked ImmunoSorbent Assay*) technique. Detection of these IgMs several days after the beginning of the symptoms makes it possible to establish a diagnosis of probability of infection with a flavivirus. Antibodies of the IgG type appear later than antibodies of the IgM type. In all cases, the search for antibodies requires two samples: one at the beginning of the clinical signs, the other 10 to 28

days later, so as to demonstrate serological conversion via an inhibition of hemagglutination reaction (IHA) or by ELISA.

Simple and inexpensive immunological tests have also been proposed, which can be used in the countries at risk and which use, as a specific immunological reagent, peptides derived from the NS1 nonstructural protein characteristic of flaviviruses. Thus, US patent 5 824 506 describes a method using peptides derived from the NS1 nonstructural protein, which makes it possible to detect the antibodies induced by the presence of the dengue virus; however, the peptides selected essentially recognize samples obtained from convalescent individuals and also recognize patients infected for the second time better than those infected for the first time; these disappointing results may be explained by the fact that the peptides used are not representative of the antigenic characteristics of the native protein and therefore lead to poor recognition of the antibodies being sought.

In all cases, only late confirmation of an infection with a flavivirus may be given.

A report from the *Sir Albert Sakzewski Virus Research Center, Royal Children's Hospital*, (A. Falconar, 1991) describes the search for the NS1 nonstructural glycoprotein in the serum of patients infected with the DEN2 virus. The authors of this report have developed a double-sandwich ELISA assay in which a rabbit serum containing polyclonal anti-NS1 antibodies, used as capture antibodies, is immobilized on a microtitration plate. The antigen captured is detected using mouse monoclonal antibodies directed against the NS1 protein, either of the dengue virus of the DEN2 type, or specific for the serological complex of dengue; the formation of the antigen/antibody complex is revealed using peroxidase-conjugated goat anti-mouse IgG. With

this method, the authors have shown, by using the degraded or purified dimeric NS1 protein as the standard, that the sensitivity of detection of the assay is approximately 4 ng/ml with the DEN2 monoclonal antibodies as the revelation probe and approximately 60 ng/ml with the group monoclonal antibodies.

However, this assay does not make it possible to detect the NS1 protein either in the case of primary infections in the acute or convalescent phase, or in secondary infections in the convalescent phase in which there is a high titer of anti-NS1 antibodies; the authors have concluded therefrom that the NS1 protein must be present in large amounts only in cases of secondary infections, this being transiently, during the infection.

Now, the inventors have developed a method for purifying the NS1 protein of a flavivirus, in the hexameric form, which has allowed them to select antibodies specific for this protein in hexameric form, and to show, surprisingly, that these antibodies are tools of choice for demonstrating the various problems of the circulating NS1 protein in the context of an infection with a flavivirus, in particular in the early phases in which the specific antibody response is undetectable, especially during primary infections with the dengue virus.

Consequently, the inventors have given themselves the aim of providing a method for the early detection of a flaviviral infection, which corresponds to practical needs better than the methods of the prior state of the art, i.e. a method which is reliable, rapid and inexpensive and which makes it possible to adapt the medical care in time.

Consequently, a subject of the present invention is a method for the early detection of a flaviviral

infection, characterized in that it comprises detecting the NS1 nonstructural glycoprotein of a flavivirus in a biological sample, throughout the duration of the clinical phase of the infection, by an immunological method using at least two antibodies, which may be identical or different,

- the first antibody or antibody for capturing the NS1 glycoprotein consisting of antibodies chosen from the group consisting of:

- polyclonal antibodies preselected by immunocapture on the NS1 protein of said flavivirus, in the hexameric form, and

- mixtures of anti-NS1 monoclonal antibodies preselected for their high affinity for the NS1 protein of said flavivirus, in the hexameric form, said monoclonal antibodies then being purified,

- the second antibody or revelation antibody being chosen from the group consisting of:

- polyclonal antibodies directed against the NS1 protein in the hexameric form, and
- a mixture of monoclonal antibodies directed against the NS1 protein in the hexameric form.

For the purpose of the present invention, the expression "hexameric form of the NS1 protein of a flavivirus" is intended to mean the native protein obtained from the culture supernatant of mammalian cells infected with said flavivirus or transformed using an expression system comprising the gene of the NS1 protein of said flavivirus, and purified according to the method of the invention as described below. This hexameric form of said NS1 protein, which differs from other forms such as the monomeric form or the dimeric form of said protein, is demonstrated using

electrophoresis or chromatography techniques such as those described in figure 1.

For the purpose of the present invention, the expression "polyclonal and monoclonal antibodies directed against the NS1 protein of a flavivirus" is intended to mean antibodies obtained by immunizing a nonhuman mammal,

- either with an NS1 protein in the hexameric form,
- or with a live or inactivated flavivirus,

said polyclonal antibodies being selected for their affinity for the NS1 protein in the hexameric form and purified in a single step, and said monoclonal antibodies being preselected for their high affinity for the NS1 protein in the hexameric form and then purified by conventional techniques, in particular by ion exchange or affinity chromatography.

For the purpose of the present invention, the expression "affinity of a monoclonal antibody for the NS1 protein in the hexameric form" is intended to mean the concentration of said protein required to saturate 50% of the sites of the antibody; this is measured by the affinity constant of said antibody, according to the protocol described in example 5.

For the purpose of the present invention, the term "high affinity" is intended to mean an affinity for which the constant is less than 10^{-8} M.

Surprisingly, the use, for detecting the NS1 protein in a biological sample, of polyclonal antibodies selected and purified by immunocapture on the NS1 protein in the hexameric form, or of monoclonal antibodies which have a high affinity for the NS1 protein in the hexameric form and which are purified, instead of a total hyperimmunized rabbit serum, makes it possible to significantly improve the sensitivity of the method and

to detect the NS1 protein circulating in the blood of patients, from the early stage of infection, both during a primary infection and a secondary infection.

The method according to the present invention has a certain number of advantages:

- it may be carried out early: the presence of the NS1 glycoprotein is revealed during the clinical phase, before the antibody response is detectable,

- it is sensitive: it is possible to detect as little as less than 1 ng of protein/ml of serum, which makes it possible to detect the circulating NS1 protein in the early phase of primary infections,

- it is rapid: an answer can be obtained within a day,

- it is relatively inexpensive and can therefore be used in the countries at risk,

- it makes it possible to distinguish vaccinated individuals from individuals recently infected with a flavivirus, since the NS1 protein will be absent in vaccinated individuals in which the antibodies may still be detectable.

According to an advantageous embodiment of said method, the flaviviral infection is an infection with the dengue virus.

According to another advantageous embodiment of said method, the first antibody is preferably attached to a suitable solid support and the second antibody is optionally conjugated to a suitable label.

According to another advantageous embodiment of said method, when the second antibody is not conjugated to a

label, its binding to the NS1 protein attached to the solid support is then detected with a third antibody, conjugated to a suitable label, said third antibody being a conventionally used antibody, such as for example an IgG directed against the second antibody and produced in particular in the goat, the pig or the donkey.

Among the labels used, mention may be made, by way of example, of fluorescent labels, the biotin/streptavidin system, nonisotopic labels or enzymes, such as for example horseradish peroxidase or alkaline phosphatase.

According to another advantageous embodiment of said method, said third antibody is conjugated to an enzyme.

According to another advantageous embodiment of said method,

- the first antibody, or capture antibody, consists of mouse polyclonal antibodies selected by immunocapture on the NS1 protein of the dengue virus, said protein being in the hexameric form, and

- the second antibody, or antibody for detecting the presence of NS1 in the biological sample to be analyzed, consists of polyclonal antibodies from a rabbit immunized with the NS1 protein of the dengue virus, said protein being in the hexameric form, the attachment of said second antibody being revealed with a third antibody, consisting of antibodies conjugated to peroxidase and directed against the second antibody.

According to another even more advantageous embodiment of said method, the mouse polyclonal antibodies are purified by immunocapture on the hexameric NS1 protein of dengue serotype 1.

A subject of the present invention is also a kit or boxed set for diagnosing a flaviviral infection, characterized in that it comprises:

- at least one capture antibody and at least one revelation antibody as defined above,

- at least one positive control consisting of the NS1 protein of a flavivirus and/or of various serotypes depending on the flavivirus, said protein being in a hexameric form, and

- at least one negative control consisting of a normal human serum.

According to an advantageous embodiment of the boxed set for diagnosis according to the invention, said NS1 protein in the hexameric form is obtained from a culture supernatant either from infected mammalian cells or from mammalian cells transfected with a recombined plasmid comprising the gene of the NS1 protein or a fragment of said gene or a fragment of the flaviviral genome, said fragments being capable of expressing all or part of the NS1 protein.

According to another advantageous embodiment of the boxed set for diagnosis according to the invention, the NS1 protein is that of the dengue virus.

According to an even more advantageous embodiment of said boxed set for diagnosis, the plasmid is the pCIneo-NS1.FGA plasmid which was deposited with the Collection Nationale de Cultures et de Microorganismes [National collection of cultures and microorganisms] held by the Institut Pasteur under the number I-2220, dated June 7, 1999.

A subject of the present invention is also a method for purifying the NS1 protein of a flavivirus, in the

hexameric form, from a culture supernatant either of infected mammalian cells or of mammalian cells transfected with a recombined plasmid comprising the gene of the NS1 protein of a flavivirus or a fragment of said gene or a fragment of the flaviviral genome, said fragments being capable of expressing the NS1 protein in a hexameric form, characterized in that, prior to the purification of the NS1 protein using conventional techniques such as affinity chromatography, the soluble form of the NS1 protein is separated from the microparticulate form of said protein, by treatment with a precipitating agent and then by centrifugation.

For example, the centrifugation is carried out at a speed greater than or equal to 10 000 g.

For the purpose of the present invention, the term "precipitating agent" is intended to mean an agent which precipitates specifically microparticulate proteins or cellular debris, such as for example polyethylene glycol, said agent being used under conventional conditions which make it possible to separate soluble proteins and microparticulate proteins or cellular debris.

In a preferred embodiment of said purification method, the hexameric NS1 protein is that of the dengue virus, in particular dengue virus serotype 1.

A subject of the present invention is also an immunogenic composition, characterized in that it comprises, as the active principle, the NS1 protein of a flavivirus, in the hexameric form, optionally associated with other proteins, in combination with at least one pharmaceutically acceptable vehicle.

In a preferred embodiment of the immunogenic composition according to the present invention, the

immunogenic composition comprises at least one mixture of the NS1 proteins in the hexameric form corresponding to the various dengue virus serotypes.

A subject of the present invention is also an immunogenic composition, characterized in that it comprises an active principle selected from the group consisting of:

- a polynucleotide capable of expressing all or part of the NS1 protein of the dengue virus, whatever its serotype,

- an expression system comprising at least one promoter capable of expressing, in the host into which it is injected, a DNA encoding the NS1 protein of the dengue virus, whatever its serotype, said DNA expressing said protein,

in combination with at least one pharmaceutically acceptable vehicle.

Vaccination protocols using nucleic acids are described in particular in international application WO 90/11092.

A subject of the present invention is the use of an NS1 protein of a flavivirus, in the hexameric form, or of a system for the expression thereof, for preparing an immunogenic composition capable of inducing the production of antibodies *in vivo*.

In a preferred method of said use, the NS1 protein is that of the dengue virus, in particular dengue virus serotype 1.

A subject of the present invention is also the use of at least one monoclonal anti-NS1 antibody having a high affinity for the NS1 protein in the hexameric form, said monoclonal antibodies then being purified, and

modified, for manufacturing a medicinal product capable of inducing passive immunization.

Advantageously, the modifications to the antibodies are, in particular, the selection of Fab fragments or the humanization of the antibodies.

A subject of the present invention is also the use of the NS1 protein in the hexameric form, for selecting *in vitro* antibodies specific for said NS1 protein, able to diagnose an infection with a flavivirus, at an early stage.

In an advantageous embodiment of said use, the antibodies are polyclonal antibodies.

In another advantageous embodiment of said use, the antibodies are monoclonal antibodies.

In another advantageous embodiment of said use, the protein is the NS1 protein of the dengue virus, in particular dengue virus serotype 1.

The anti-NS1 monoclonal antibodies are advantageously obtained by fusing spleen cells from a mouse immunized with the NS1 protein in the hexameric form, with suitable myeloma cells.

A subject of the present invention is also a method for expressing a polynucleotide encoding the NS1 protein of a dengue virus, characterized in that it comprises the expression of a polynucleotide as defined in the sequence SEQ ID No. 1, associated with a promoter for said polynucleotide, in suitable eukaryotic cells.

Other characteristics and advantages of the invention appear in the remainder of the description and the examples illustrated by the figures in which:

- Figure 1 represents the purified hexameric extracellular NS1 protein obtained after exclusion chromatography. (**a**) After exclusion chromatography, the protein is concentrated to 0.5 mg/ml by ultrafiltration and treated with dimethyl suberimidate (DMS) at 0, 0.5, 5 and 50 mM. The products obtained are placed in a nonreducing Laemmli buffer, separated on a 4 to 20% gradient acrylamide gel and stained with Coomassie blue. A sample treated with 50 mM DMS is heated for 3 min at 95°C before electrophoresis in order to dissociate the noncovalent oligomers. (**b**) The purified NS1 protein is treated overnight at 37°C with 0.5% or 1% of *n*-octylglucoside (nOG) and, optionally, treated with 25 mM of DMS for 1 hour. The proteins are separated without heat denaturation on a 4 to 20% gradient acrylamide gel and detected via *immunoblotting* with a monoclonal anti-NS1 antibody from the literature or as defined above.
- Figure 2 represents the sequence of the NS1 protein of dengue virus serotype 1, obtained with clone 4C of example 2 below, and also the corresponding coding sequence.
- Figure 3 illustrates the results obtained by assaying the circulating NS1 protein using the method of detection by capture-ELISA in patients infected beforehand with a dengue virus, whose sera were taken during the acute and convalescent phases, and also the comparison with the results obtained using the techniques of the prior art, IHA (inhibition of hemagglutination of dengue virus serotypes 1, 2, 3 or 4) and MAC ELISA (*immunoglobulin M Antibody Capture Enzyme-Linked ImmunoSorbent Assay*); D1 corresponds to dengue serotype 1; D2 corresponds to dengue serotype 2; D3 corresponds to dengue serotype 3 and D4 corresponds to dengue serotype 4; ID = patient's identity; 1 corresponds to the first sample in the

acute phase of the disease, 2 corresponds to the second sample in the convalescent phase (taken 2 to 4 weeks after the first); in the capture-ELISA assay, the values are expressed as optical density obtained for the same serum diluted 10, 30 or 90 times.

- Figure 4 illustrates the detection of the NS1 protein using the capture-ELISA assay on sera from patients infected with dengue virus serotype 1 from French Guiana. The numbers indicated represent the number of patients divided up per category (positivity or negativity by capture-ELISA and positivity or negativity for IgM).
- Figure 5 illustrates the results obtained for 4 patients from French Guiana infected with dengue virus 1, from whom samples were taken daily during the clinical phase of the disease from D1 to D5. Each graph corresponds to a patient with, for each day on which a sample was taken, both the results of detection of the NS1 protein with the capture-ELISA assay developed, the results of RT-PCR and the results obtained using the MAC-ELISA technique. The O.D. values reported were corrected for once the value of the background noise. The positivity thresholds are indicated by the broken lines.
- Figure 6 indicates the characteristics of the anti-NS1 monoclonal antibodies F22 and G18.
- Figure 7 illustrates the detection of the NS1 protein with the capture-ELISA assay using the monoclonal approach in comparison with the capture-ELISA assay using the polyclonal approach as described in example 3. The results obtained are reported in the form of optical density values measured for each dilution of serum analyzed (10th, 30th or 90th) and less the mean value of the negative controls.
- Figure 8 illustrates the demonstration of the NS1 protein in the sera from patients infected with

the yellow fever virus, using an capture-ELISA assay specific for yellow fever. For each serum tested, the optical density value measured using the assay developed, less the mean value of the negative controls, the optical density value measured using the MAC-ELISA assay specific for yellow fever IgMs and the results of the viral isolation, when they are available, are reported.

Example 1: Purification of the NS1 protein of dengue virus serotype 1

1. Materials and methods

The protein is produced on Vero cells infected with dengue virus serotype 1, strain FGA/89 (P. Després *et al.*, *Virol*, (1993), **196**, 209-219), under the conditions adapted for the method described by A.K.I. Falconar *et al.*, (*J. Virol. Meth,* (1990), **30**, 323-332).

The culture medium is harvested 5 days after infection and centrifuged at 1500 g to remove the cellular debris. The centrifugation supernatant is brought to 20 mM Tris-HCl, 1 mM sodium azide, and concentrated 6 times by cold ultrafiltration, and the viral particles are removed by precipitation for 2 h at 4°C, with a concentration of 7.5% of polyethylene glycol (PEG), followed by centrifugation for 30 minutes at 10 000 g. The clarified supernatant, containing 7.5% PEG is treated with 0.05% Tween 20 and 1 mg/ml of aprotinin, and then passed over an immunoaffinity column to which is attached an anti-NS1 monoclonal antibody. The protein is eluted according to the diethylamine technique, as described in Falconar *et al.*, (mentioned above), and concentrated by ultrafiltration, and the elution solution is exchanged with a 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM of sodium azide.

2. Results

The results are illustrated in figure 1.

Figure 1a shows that the NS1 protein is indeed in the hexameric form. The proportion of hexameric form increases with an increase in concentration of DMS (figure 1a).

The extracellular NS1 protein in the hexameric form may be transformed into dimeric subunits in the presence of the nonionic detergent *n*-octylglucoside (nOG) (figure 1b). After incubation overnight at 37°C in the presence or absence of n-octylglucoside (nOG) and treatment with 25 mM DMS, it is observed that, in the absence of nOG, bands are present which correspond to the dimer, to the tetramer and to the hexamer, and that, in the presence of nOG, there is partial or complete dissociation of the hexamer depending on the concentration of nOG (figure 1b).

Example 2: Expression of the NS1 protein of dengue virus serotype 1 by Vero cells

1. Materials and methods

The pCIneo-NS1.FGA plasmid (deposited with the Collection Nationale de Cultures et de Microorganismes [National collection of cultures and microorganisms] (CNCM) of the Institut Pasteur under the No. I-2220, dated June 7, 1999) containing the gene of the NS1 protein comprising the gene encoding its signal peptide, preceded by a translation initiation codon and followed by a translation termination codon, is introduced into the competent bacterium *Escherichia coli* (epicurian SURE from Stratagène). This plasmid is amplified in bacterial culture and purified according to the conventional technique for preparing plasmid DNA. The purified DNA is used to sequence various clones (the sequence of clone 4C is illustrated in figure 2) and to transfect Vero cells using either a

suitable mixture with cationic liposomes, such as DOTAP (Boehringer Mannheim), or with a nonliposomal agent, such as FuGENE (Boehringer Mannheim). The FuGENE and the DNA are pre-incubated in medium without serum for 15 min, and then the mixture is brought into contact with a layer of Vero cells for 24 h. The cells are then rinsed with PBS (phosphate buffered saline), fixed for 20 min at room temperature with a solution of PBS containing 3% of paraformaldehyde and permeabilized for 5 min with PBS containing 0.5% Triton X-100. The presence of NS1 antigen is then revealed using specific antibodies, which are recognized by a fluorescein-labeled conjugated antibody.

2. Results

It is thus possible to demonstrate a strong fluorescent signal, specific for the NS1 viral antigen, in approximately 20% of the transfected cells.

The expression of NS1 is thus demonstrated and stable lines may be established in the presence of neomycin, which is a selection marker for the transfected cells.

Figure 2 illustrates the sequence of the NS1 protein of dengue virus serotype 1 thus obtained, and also the corresponding coding sequence.

Example 3: Implementation of the capture-ELISA technique according to the invention in the context of an infection with dengue virus serotype 1 and comparison with the methods of the prior state of the art

1. Principle of the capture-ELISA technique

The NS1 viral antigen is captured using monospecific mouse polyclonal antibodies purified beforehand by

immunocapture on the purified hexameric NS1 protein of dengue serotype 1.

The presence of NS1 is revealed using antibodies from rabbits immunized with the purified hexameric NS1 protein, themselves recognized by antibodies conjugated to horseradish peroxidase.

2. Materials and methods

a- Purification of mouse polyclonal antibodies directed against the NS1 protein

a_1- Attachment of the NS1 protein to a membrane

The purified NS1 protein is attached by adsorption to an amphoteric nylon membrane (Nytran, Schleicher & Schuell). The surface of the membrane is then saturated with bovine albumin present at a concentrate of 3% in a phosphate buffered saline solution (PBS; 10 mM phosphate; pH 7.2; 150 mM NaCl). After 2 rinses in PBS, the membrane is treated with PBS containing 0.25% of glutaraldehyde for 15 min at room temperature. After 3 rinses in PBS, the membrane is neutralized with a 100 mM glycine buffer containing 3% bovine albumin, rinsed twice in PBS and then stored at 4°C in PBS with 1 mM sodium azide.

a_2- Purification of the mouse monospecific polyclonal antibodies (capture antibodies)

- production of polyclonal ascites: the brains of young Swiss mice infected with dengue virus and moribund are ground in 9 ml of PBS buffer. The product is centrifuged for 10 min at 10 000 g at 4°C.

The viral suspension is injected into Swiss mice, according to the following calendar of events:

- D0: 0.5 ml of antigen subcutaneously into the thigh,

- D3: 0.4 ml of antigen and 0.1 ml of complete Freund's adjuvant intraperitoneally,
- D25: 0.5 ml of antigen intraperitoneally,
- D26: 0.5 ml of TG180 mouse ascites, and
- D28: 0.5 ml of antigen intraperitoneally.

The ascites are harvested on D42.

After having collected the ascites, the coagulum is allowed to form for 1 hour at room temperature and then centrifugation is carried out for at least 30 min at 1500 g. The supernatant is left to stand overnight at 4°C. The pH of the supernatant is adjusted to 4.8 with 2M acetic acid and the supernatant is then centrifuged again under the same conditions. The pH of the supernatant is then brought to 7.0-7.2 by adding a 2N sodium hydroxide solution. The supernatant may be stored at -20°C.

- purification of the mouse antibodies specific for dengue virus serotype 1:

The membrane is incubated for one hour at room temperature in a mixture of polyclonal ascites directed against the 4 dengue virus serotypes prepared as described above.

After rinsing the membrane 3 times in PBS, the antibodies attached to the NS1 protein are eluted with a diethylamine solution, pH 11.4 (Dubelco medium modified with Iscove (Gibco) containing 100 mM diethylamine). The antibodies are concentrated by ultrafiltration and returned to a PBS buffer containing 1 mM sodium azide.

b- Preparation of rabbit polyclonal antibodies directed against the NS1 protein (revelation antibodies):

The rabbits were immunized with 3 or 4 successive injections of 30 µg of hexameric NS1 protein purified

according to the method of example 1, given on D0, D7, D21 and, optionally, on D49, and followed by bleeding out on D83. The serum is depleted of nonspecific signal by incubation with Sepharose beads bearing a monoclonal antibody described in the literature or prepared as described above.

c- Capture-ELISA method

c_1- Standard curve

For each capture-ELISA plate intended for testing human sera, a standard range is prepared from a solution of NS1 protein purified according to the method described in point 1, the initial concentration of which is 0.5 µg/ml, and which is diluted in 3-fold serial dilutions.

C_2- Detection of the circulating NS1 protein during the acute phase:

The purified mouse polyclonal antibodies obtained according to the method described above (capture antibodies) are attached to a plate, diluted in a PBS solution and left to incubate overnight at 4°C. After 3 rinses for 5 minutes with a solution of PBS/0.05% Tween, the plate is saturated with a mixture of PBS, 0.05% Tween and 3% milk for 30 minutes at room temperature. After 3 rinses with a solution of PBS/0.05% Tween, the sera to be tested, diluted or undiluted, are deposited and left to react for one hour, still at room temperature. The 1/10th, 1/30th and 1/90th dilutions are prepared in a solution of PBS/0.05% Tween. After 3 rinses, the second antibody specific for NS1 (revelation antibody obtained in point 3 above) is added, after having been diluted in a mixture of PBS/0.05% Tween and of 3% milk, and left to incubate for 45 minutes at 37°C. After 3 rinses, the anti-IgG antibodies are directed against the second antibody and labeled with peroxidase, said antibody

being prepared under conventional conditions known to those skilled in the art, is added and the incubation is carried out for 45 minutes at 37°C. After 3 rinses, revelation is carried out for 10 minutes with a solution of TMB (3,3', 5,5'-tetramethylbenzidine, Kierkegaard & Perry Lab). The colorimetric reaction is stopped with sulfuric acid.

3. Results

They are illustrated in figure 3.

The capture-ELISA technique according to the invention makes it possible to detect the presence of NS1 protein in the acute phase of the disease, this detection being independent of whether the patients have a primary or secondary infection.

The results confirm that the presence of the NS1 protein is transient, since this protein is not detected in the samples taken in the convalescent phase (figure 3).

93% of the samples taken in the acute phase of the disease prove to be negative using the MAC ELISA assay, whereas 100% of the samples taken in the convalescent phase prove to be positive in this same assay (figure 3).

Similarly, the inhibition of hemagglutination assay (IHA) does not make it possible to detect infection with dengue virus serotype 1 in 80% of cases in the acute phase of the disease, but this test proves to be positive in 100% of the samples taken in the convalescent phase (figure 3). According to the WHO criteria, an IHA level of less than 1280 in the serum taken in the convalescent phase allows diagnosis of a primary dengue infection and a level of greater than 1280 allows diagnosis of a secondary dengue infection.

Half of the positive sera in this study therefore correspond to cases of primary dengue and the other half to cases of secondary dengue. The capture-ELISA technique according to the invention thus makes it possible to detect the NS1 protein in cases of primary and secondary dengue.

Example 4: Determination of the detection window

1. Materials and methods

a- Study carried out on a population of patients from French Guiana infected with dengue virus 1

The samples are taken from patients infected with dengue virus serotype 1, between D0, marking the appearance of the clinical signs (initially a nondifferentiated fever) and D66 corresponding to the end of the convalescent phase.

The presence of circulating NS1 is sought in the sera of these patients, according to the capture-ELISA method described in example 3, and the result obtained is compared with the positivity for specific IgMs measured by MAC-ELISA, when the data are available.

b- Daily monitoring of 4 patients infected with dengue virus 1

Samples were taken daily from 4 patients during the clinical phase from D1 to D5. An RT-PCR reaction to reveal the viral RNA, a MAC-ELISA assay to detect IgMs specific for dengue virus and a search for the dengue NS1 antigen according to the capture-ELISA method described above were carried out on each blood sample.

2. Results

a- Determination of the detection window

The results are given in figure 4.

Between D1 and D6, the possibility of detecting the circulating NS1 protein oscillates between 64% (on D2) and 100% (on D5) of the infected patients. Beyond D10, the circulating NS1 protein is no longer detected, whereas the antibody response becomes predominant.

Detection of the circulating NS1 protein does not appear to be dependent on the presence of total IgMs (specific for the viral antigens) which appear, in certain cases, on D3 and culminate from D5, nor even, for certain patients, on the presence of total IgGs which may appear from D2. On the other hand, the absence of detection of the NS1 antigen in clinical phase sera may be explained by the presence of IgGs specifically directed against NS1.

Thus, the detection window for the NS1 antigen in the serum, using the capture-ELISA technique according to the present invention, is preferably between D1 and D6 after appearance of the clinical signs.

b- Daily monitoring of 4 patients infected with dengue virus

The results are given in figure 5.

In the 4 patients studied, the NS1 protein is detected continuously up to D5, this being regardless of the day on which the sample was taken relative to the start of symptoms. For certain patients, the detection window for the protein is wider than the period of viremia, detected by RT-PCR.

Example 5: Implementation of the capture-ELISA technique with monoclonal tools in the context of an infection with dengue virus serotype 1 and comparison with the capture-ELISA technique described above

1. Materials and methods

a- Production and characterization of mouse monoclonal antibodies directed against the NS1 protein of dengue virus serotype 1

a_1- Production of mouse monoclonal antibodies directed against the NS1 protein

Female Balb/C mice were immunized with 7 injections of 10 µg of hexameric NS1 protein of dengue virus serotype 1, purified according to the method of example 1. The first injection in complete Freund's adjuvant and the subsequent five injections in incomplete Freund's adjuvant are given subcutaneously 15 days apart. The final injection, in incomplete Freund's adjuvant, given three days before the animal is sacrificed, is given intraperitoneally.

The cells from the spleen of the immunized mice are fused with the murin myeloma and cultured until clones appear, according to standard protocol.

a_2- Identification of hybridomas secreting anti-NS1 antibodies

Antibodies specific for the NS1 protein were detected either using a conventional ELISA technique or using a capture-ELISA technique.

-Conventional ELISA technique

The hexameric NS1 protein purified according to the method of example 1 is attached to a plate by adsorption, at the concentration of 1 µg/ml in a PBS solution overnight at 4°C. After 3 washes with a

solution of PBS/0.1% Tween (PT), the protein is incubated with the supernatants of the various hybridomas diluted two-fold with a solution of PT containing 0.5% gelatin (PTG), for 1 h at 37°C. After 3 washes with PT, the peroxidase-labeled anti-mouse IgG antibody diluted in PTG is added and incubated for 1 h at 37°C. After 3 washes, revelation is carried out with a solution of hydrogen peroxide in the presence of orthophenylenediamine.

-Capture-ELISA technique

The technique used is described in example 3 (cf. detection of the circulating NS1 protein in the acute phase), but replacing the dilutions of sera to be tested with a 1/10th dilution, in the PTG solution, of culture supernatant on uninfected Vero cells or Vero cells infected for 5 days with dengue virus serotype 1, and precipitated with 7% of polyethylene glycol (cf. example 1: purification of the NS1 protein of dengue virus serotype 1). The reactivity of the supernatants from the various hybridomas with respect to the culture supernatant from uninfected Vero cells is used as a control for nonspecific signal.

a_3- Reactivity of the anti-NS1 monoclonal antibodies, by indirect immunofluorescence, on Vero cells infected with one of the 4 dengue virus serotypes

The Vero cells are infected for 40 h with one of the 4 dengue virus serotypes:

serotype 1: strain FGA/89
serotype 2: strain NG
serotype 3: strain H87
serotype 4: strain H241

After 1 wash with a solution of PBS, the cells are fixed with a solution of 3% paraformaldehyde in PBS for 30 minutes at laboratory temperature. The cells rinsed in PBS are then permeabilized with a solution of 0.5%

Triton X-100 in PBS for 10 minutes. After rinsing in PBS, the cells are incubated for 1 h with the supernatants from the various hybridomas which have reacted positively by ELISA. After 3 washes with PBS, the fluorescene-labeled anti-mouse IgG antibody is added and incubated for 1 h. After 3 washes in PBS, the slides are covered with a coverslip and observed under a fluorescent microscope.

a_4- Preparation of the mouse monoclonal ascites

The monoclonal ascites are produced in Balb/C mice. The mice are given an intraperitoneal injection of 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane, Sigma) one week before the intraperitoneal injection of the hybridoma clone secreting the monoclonal antibody. The ascites are removed as they form, centrifuged at 1500 rpm for 20 minutes and stored at -20°C.

a_5- Determination of the isotype of the anti-NS1 monoclonal antibodies

The isotype of the anti-NS1 antibody is determined by ELISA using antibodies directed against the various murine immunoglobulin subclasses: IgG1, IgG2a, IgG2b and IgG3. The light chain of the immunoglobulin is determined according to an identical methodology.

a_6- Determination of the affinity constant of the anti-NS1 monoclonal antibodies (B Friguet et al., *J. Immunol*, (1985), **77**, 305-319)

The affinity of an antibody corresponds to the concentration of antigen required to saturate 50% of the sites of the antibodies. An incubation is carried out in liquid medium between the antibody at constant concentration and the antigen at decreasing concentration overnight at 4°C in order to reach the equilibrium of the reaction. The concentration of free

antibodies, after equilibrium, is determined using an ELISA assay: the mixture is deposited onto a plate preincubated with the antigen. After incubation for 20 minutes at 4°C (to avoid a shift of the equilibrium), the ELISA is revealed with a β-galactosidase-coupled anti-mouse IgG, followed by the enzymatic reaction. The dissociation constant KD is then determined.

a_7- Competition reaction for the various anti-NS1 monoclonal antibodies

This reaction makes it possible to determine the specificity of the monoclonal antibodies with respect to the same epitope or to different epitopes. Epitope determination brings into play the reactivity for an antigen, of an unlabeled monoclonal antibody and of a second monoclonal antibody, coupled to biotin.

The first monoclonal antibody, unlabeled, is placed at saturating concentration (determined beforehand by ELISA) on a plate to which the antigen has been attached beforehand, and incubated for 2 h at 37°C. After 4 washes in PT solution at 4°C, the second monoclonal antibody, coupled to biotin, is added and incubated for 20 minutes at 4°C. After 4 washes in PT solution at 4°C, the solution of peroxidase-labeled streptavidin conjugate is added and incubated for 1 h at 37°C. After 4 washes in PT solution, the complex is revealed with a solution of hydrogen peroxide in the presence of orthophenylenediamine. If a signal is obtained after reading on a spectrophotometer, this indicates that the epitopes recognized by the 2 antibodies are different. If the opposite is true, the 2 monoclonal antibodies are directed against the same epitope of the antigen.

<u>b- purification of the monoclonal antibodies G18 and F22</u>

The antibodies G18 and F22 are purified by immunoaffinity as described in Exmaple 3.

c- detection of the circulating NS1 protein with a capture-ELISA assay using the monoclonal antibodies

The purified monoclonal antibodies G18 and F22 are mixed in a solution of PBS at a given dilution and incubated overnight at 4°C. The subsequent steps of this ELISA assay are similar to those of the previous example.

d- comparison of the capture-ELISA assay using the monoclonal approach with that using the polyclonal approach

A panel of serum from French Guiana was tested on the same day with the capture-ELISA assay using the monoclonal approach and then using the polyclonal approach. The sera are tested at various dilutions: 10th, 30th and 90th.

2. Results

a- Characteristics of the monoclonal antibodies

The results are given in figure 6.

The antibodies G18 and F22 were selected for their ability to bind, with high affinity, to different epitopes of the NS1 protein. The antibody F22 is specific for dengue virus serotype 1, and G18 is specific for dengue virus serotypes 1 and 3.

b- Use of the monoclonal antibodies for NS1 antigen capture

The results are given in figure 7.

The monoclonal antibodies selected not only reproduce the results obtained with the polyclonal approach, but they exhibit more marked reactivities than the polyclonal antibodies. The monoclonal tool developed therefore appears to be particularly suitable for the diagnostic use which must be made of it.

Example 6: Implementation of the capture-ELISA technique according to the invention in the context of an infection with another dengue virus serotype or another flavivirus

1. Materials and methods

a- Preparation of culture supernatants

The Vero cells are infected either with dengue virus 2 or with the Japanese encephalitis virus or the yellow fever virus. The culture supernatants are then prepared according to the method described in example 1.

b- Purification of the monoclonal antibodies directed against the NS1 protein of the yellow fever virus and of the Japanese encephalitis virus

The monoclonal ascites of the antibodies 8G4, 1A5 and 2D10 (J.J. Schlesinger *et al.*, *Virology*, (1983), **125**, 8-17) directed against the NS1 protein of the yellow fever virus, and of the antibodies 171-2-2 and 70-14-20 directed against the NS1 protein of the Japanese encephalitis virus, are purified on protein A Sepharose CL-4B beads (Pharmacia Biotech). These monoclonal ascites are incubated overnight at 4°C on the protein A beads. After the beads have been rinsed 3 times in PBS/0.05% Tween, the antibodies attached to the protein A beads are eluted with a solution of glycine buffer, pH=3. They are then concentrated by ultrafiltration and returned to a PBS buffer containing 1 mM sodium azide.

c- Detection of the NS1 protein in the dengue virus 2 culture supernatants

c_1- Antibodies used

The capture step is carried out with a mixture of ascites of the monoclonal antibodies 3D1.4 and 1A12 (A.K.I. Falconar *et al., Arch. Virology*, (1994), **137**, 315-326). The protein is then recognized with a mixture of two rabbit antibodies: the serum obtained after immunization with the purified protein described in example 3 and a rabbit serum obtained after immunization with the viruses of the four dengue serotypes.

c_2- Capture-ELISA method

The technique used is the same as that described in example 3.

d- Detection of the NS1 protein in the Japanese encephalitis virus culture supernatants

d_1- Antibodies used

The purified monoclonal antibodies 171-2-2 and 70-14-20 are used for the capture step. The protein is then recognized with a mixture of two sera from rabbits which have been immunized beforehand with recombined proteins of the NS1 protein of Japanese encephalitis.

d_2- Capture-ELISA method

The technique used is the same as that described in example 3.

e- Detection of the NS1 protein in the yellow fever virus culture supernatants and the sera from patients infected with this virus

e_1- Antibodies used

The purified monoclonal antibodies 8G4, 1A5 and 2D10 are mixed, at a given dilution, in a solution of PBS and used as capture antibodies. The second antibody specific for yellow fever NS1 used originates from a serum of a rabbit immunized beforehand against the NS1 protein of the yellow fever 17D virus (J.J. Schlesinger *et al., J. immunol.* (1985), **135**, 2805-2809).

e_2- Capture-ELISA method

The technique used is the same as that described in example 3.

2. <u>Results</u>

Secretion of the NS1 protein has previously been reported in *in vitro* cell cultures infected with various flaviviruses, the DEN2 virus (Winkler *et al., Virology* (1988), **162**, 187-196, Pryor *et al., Virology* (1993) **194**, 769-780), the tick-borne encephalitis virus (Lee *et al., J. Gen. Virol.* (1989), **70**, 335-343, Crooks *et al., J. Chrom.* (1990), **502**, 59-68, Crooks *et al., J. Gen. Virol.* (1994), **75**, 3453-3460), the Japanese encephalitis virus (Mason, *Virology* (1989), **169**, 354-364, Fan *et al., Virology* (1990), **177**, 470-476), the Murray valley encephalitis virus (Hall *et al., J. Virol. Meth.* (1991), **32**, 11-20) and the yellow fever virus (Post *et al., Vir. Res.* (1990), **18**, 291-302). As these results were obtained using different ELISA techniques, we sought to demonstrate the protein, using the capture-ELISA technique of the present invention, in supernatants of infected mammalian cells.

The NS1 protein is detectable in the culture supernatants of the Vero cells infected either with the

DEN2 virus, with the Japanese encephalitis virus or with the yellow fever virus.

It was also possible to demonstrate the protein, using this technique, in sera from patients infected with the yellow fever virus, as demonstrated by the results given in figure 8. Among the 18 sera generously provided by Ch. Mathiot (Institut Pasteur of Dakar), 7 are positive by NS1 antigenemia, and, as for the DEN1 virus, detection of the circulating NS1 protein appears to be indifferent to the presence of IgMs specific for yellow fever.

The capture-ELISA technique according to the present invention makes it possible to detect the NS1 protein in the culture supernatants of cells infected with various flaviviruses and in the sera from patients infected with the yellow fever virus. Because of this, it may have a diagnostic application for detecting an infection with a flavivirus other than the DEN1 virus.

CLAIMS

1. A method for the early detection of a flaviviral infection, characterized in that it comprises detecting the NS1 nonstructural glycoprotein of a flavivirus in a biological sample, throughout the duration of the clinical phase of the infection, by an immunological method using at least two antibodies, which may be identical or different,

- the first antibody or antibody for capturing the NS1 glycoprotein consisting of antibodies chosen from the group consisting of:
- polyclonal antibodies preselected by immunocapture on the NS1 protein of said flavivirus, in the hexameric form, and

- mixtures of anti-NS1 monoclonal antibodies preselected for their high affinity for the NS1 protein of said flavivirus, in the hexameric form, said monoclonal antibodies then being purified,

- the second antibody or revelation antibody being chosen from the group consisting of:

- polyclonal antibodies directed against the NS1 protein in the hexameric form, and

- a mixture of monoclonal antibodies directed against a NS1 protein in the hexameric form.

2. The detection method as claimed in claim 1, characterized in that the flaviviral infection is an infection with the dengue virus.

3. The detection method as claimed in either of claims 1 and 2, characterized in that the first antibody is attached to a suitable solid support

and the second antibody is optionally conjugated to a suitable label.

4. The detection method as claimed in claim 3, characterized in that when the second antibody is not conjugated to a label, its binding to the NS1 protein attached to the solid support is then detected with a third antibody, conjugated to a suitable label.

5. The detection method as claimed in claim 4, characterized in that the third antibody is conjugated to an enzyme.

6. The detection method as claimed in claim 5, characterized in that:

- the first antibody, or capture antibody, consists of mouse polyclonal antibodies selected by immunocapture on the NS1 protein of the dengue virus, said protein being in the hexameric form, and

- the second antibody, or antibody for detecting the presence of NS1 in the biological sample to be analyzed, consists of polyclonal antibodies from a rabbit immunized with the NS1 protein of dengue virus serotype 1, said protein being in the hexameric form, the attachment of said second antibody being revealed with a third antibody, consisting of antibodies conjugated to peroxidase and directed against the second antibody.

7. A boxed set for the early diagnosis of a flaviviral infection, characterized in that it comprises:

- at least one capture antibody and at least one revelation antibody as defined in any one of claims 1 to 6,

- at least one positive control consisting of the NS1 protein of a flavivirus and/or of various serotopes depending on the flavivirus, said protein being in the hexameric form, and

- at least one negative control consisting of a normal human serum.

8. The boxed set for diagnosis as claimed in claim 7, characterized in that said NS1 protein is obtained from a culture supernatant either from infected mammalian cells or from mammalian cells transfected with a recombined plasmid comprising the gene of the NS1 protein of a flavivirus or a fragment of said gene or a fragment of the flaviviral genome, said fragments being capable of expressing all or part of the NS1 protein.

9. The boxed set for the early diagnosis of a flaviviral infection as claimed in either of claims 7 and 8, characterized in that the NS1 protein is that of the dengue virus.

10. The boxed set for the early diagnosis of a flaviviral infection as claimed in either of claims 8 and 9, characterized in that said plasmid was deposited with the Collection Nationale de Cultures et de Microorganismes [National collection of cultures and microorganisms] held by the Institut Pasteur under the number I-2220, dated June 7, 1999.

11. A method for purifying the NS1 protein of a flavivirus, in the hexameric form, from a culture supernatant either of infected mammalian cells or of mammalian cells transfected with a recombined plasmid comprising the gene of the NS1 protein or a fragment of said gene or a fragment of the

flaviviral genome, said fragments being capable of expressing the NS1 protein, characterized in that, prior to the purification of the NS1 protein using conventional techniques, it comprises a step for separating the soluble form of the NS1 protein from the microparticulate form of said protein, by treatment with a precipitating agent and then by centrifugation.

12. An immunogenic composition, characterized in that it comprises, as the active principle, the NS1 protein of a flavivirus, in the hexameric form, optionally associated with other proteins, in combination with at least one pharmaceutically acceptable vehicle.

13. The immunogenic composition as claimed in claim 12, characterized in that it comprises at least one mixture of the NS1 proteins in the hexameric form corresponding to the various dengue virus serotypes.

14. The use of the NS1 protein in the hexameric form, or of a system for the expression thereof, for preparing an immunogenic composition capable of inducing the production of antibodies *in vivo*.

15. The use of at least one monoclonal anti-NS1 antibody having a high affinity for the NS1 protein in the hexameric form, said form being nondegraded, said monoclonal antibodies then being purified and modified, for manufacturing a medicinal product capable of inducing passive immunization.

16. The use of the NS1 protein in the hexameric form, said form being nondegraded, for selecting *in vitro* specific anti-NS1 antibodies able to

diagnose an infection with a flavivirus, at an early stage.

17. An immunogenic composition, characterized in that it comprises an active principle selected from the group consisting of:

- a polynucleotide capable of expressing all or part of the NS1 protein of the dengue virus, whatever its serotype,

- an expression system comprising at least one promoter capable of expressing, in the host into which it is injected, a DNA encoding the NS1 protein of the dengue virus, whatever its serotype, said gene expressing said protein,

in combination with at least one pharmaceutically acceptable vehicle.

18. A method for expressing a polynucleotide encoding the NS1 protein of a dengue virus, characterized in that it comprises the expression of a polynucleotide as defined in the sequence SEQ ID No. 1, associated with a promoter for said polynucleotide, in suitable eukaryotic cells.

19. The method for purifying the NS1 protein as claimed in claim 11, characterized in that the flavivirus is a dengue virus, whatever its serotype.

20. The method for purifying the NS1 protein as claimed in claims 11 and 19, characterized in that the flavivirus is a dengue virus serotype 1.

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(54) Title: EARLY DETECTION OF FLAVIVIRUSES USING THE NS1 GLYCOPROTEIN

(54) Titre: DETECTION PRECOCE DES FLAVIVIRUS EN UTILISANT LA CLYCOPROTEINE NS1

(57) Abstract: The invention concerns a method for early detection of a flavivirus-induced infection, comprising the detection of the flavivirus non-structural glycoprotein NS1 in a biological sample during the clinical phase of the infection, by an immunological method using at least two identical or different antibodies, the first antibody consisting of polyclonal or monoclonal antibodies preselected for their high affinity for said NS1 protein hexameric in shape.

(57) Abrégé: Méthode de détection précoce d'une infection flavivirale, comprenant la détection de la glycoprotéine non-structurale NS1 d'un flavivirus dans un échantillon biologique, pendant la phase clinique de l'infection, par une méthode immunologique mettant en oeuvre au moins deux anticorps identiques ou différents, le premier anticorps étant constitué par des anticorps polyclonaux ou monoclonaux préalablement sélectionnés pour leur affinité élevée pour ladite protéine NS1 de forme hexamérique.

WO 00/75665 A1

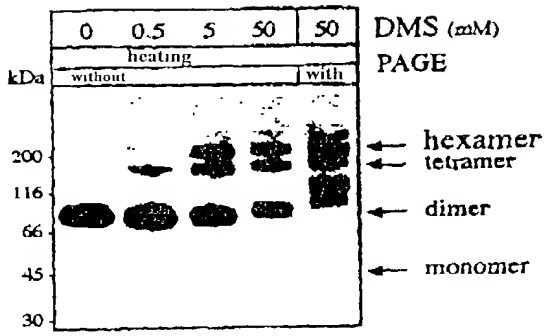
0
0.5
5
50
50
DMS (mM)
heating
without
with
PAGE
kDa
200
116
66
45
30
hexamer
tetramer
dimer
monomer

FIG. 1a

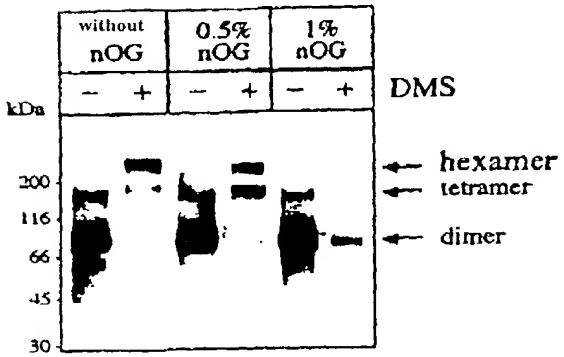
without nOG
0.5% nOG
1% nOG
−
+
−
+
−
+
DMS
kDa
200
116
66
45
30
hexamer
tetramer
dimer

FIG. 1b

SEQ ID N° 1 : NS1 protein of dengue virus serotype 1

```
1/1                                                                  31/11
atg agg agc gcg tcg ctt tcg atg acg tgc att gca gtt ggc atg gct aca ctg tac cta
Met arg ser ala ser leu ser met thr cys ile ala val gly met val thr leu tyr leu
61/21                                                                91/31
gga gtc acg gtt caa gcg gac tcg gga tgt gta atc aac tgg aag ggc aga gaa ctc aaa
gly val met val gln ala asp ser gly cys val ile asn trp lys gly arg glu leu lys
121/41                                                               151/51
tgt gga agt ggc att ttt gtc act aat gaa gtc cac act tgg aca gag caa tac aaa ttc
cys gly ser gly ile phe val thr asn glu val his thr trp thr glu gln tyr lys phe
181/61                                                               211/71
cag gct gac tcc cca aaa aga ctg tca gca gcc att ggg aag gca tgg gag gag ggc gtg
gln ala asp ser pro lys arg leu ser ala ala ile gly lys ala trp glu glu gly val
241/81                                                               271/91
tgt gga atc cga tca gcc acg cgt ctt gag aac atc atg tgg aag caa ata tca aat gaa
cys gly ile arg ser ala thr arg leu glu asn ile met trp lys gln ile ser asn glu
301/101                                                              331/111
ctg aac cac atc cta ctt gaa aat gac atg aaa ttc aca gtg gtt gta gga gat gct aat
leu asn his ile leu leu glu asn asp met lys phe thr val val val gly asp ala asn
361/121                                                              391/131
gga att ttg gcc cag ggg aaa aaa atg atc agg cca caa ccc atg gaa cac aaa tac tca
gly ile leu ala gln gly lys lys met ile arg pro gln pro met glu his lys tyr ser
421/141                                                              451/151
tgg aaa agc tgg gga aaa gcc aag atc ata gga gca gac aca cag aac acc acc ttc atc
trp lys ser trp gly lys ala lys ile ile gly ala asp thr gln asn thr thr phe ile
481/161                                                              511/171
atc gac ggc cca gac act cca gaa tgc ccc gat gac caa aga gcg tgg aac att tgg gaa
ile asp gly pro asp thr pro glu cys pro asp asp gln arg ala trp asn ile trp glu
541/181                                                              571/191
gtt gag gac tat ggg ttt gga att ttc acg aca aac ata tgg ctg aaa ttg cgt gac tcc
val glu asp tyr gly phe gly ile phe thr thr asn ile trp leu lys leu arg asp ser
601/201                                                              631/211
tac acc caa atg tgt gac cac cgg cta atg cca gct gcc gtc aag gac agc aag gca gtt
tyr thr gln met cys asp his arg leu met ser ala ala val lys asp ser lys ala val
661/221                                                              691/231
cat gct gac atg ggg tac tgg ata gaa agt gaa aag aac gag acc tgg aag cta gcg aga
his ala asp met gly tyr trp ile glu ser glu lys asn glu thr trp lys leu ala arg
721/241                                                              751/251
gcc tcc tcc aca gaa gcc aag aca tgc att tgg ccg aaa tcc cac act cta tgg agt aat
ala ser phe ile glu val lys thr cys ile trp pro lys ser his thr leu trp ser asn
781/261                                                              811/271
gga gtt ttg gaa agt gaa atg ata atc cca aag ata tat gga gga cca ata tct cag cac
gly val leu glu ser glu met ile ile pro lys ile tyr gly gly pro ile ser gln his
841/281                                                              871/291
aat tac aga cca ggg tat ttc aca caa aca gca ggg cca tgg cac cta ggt aag ttg gaa
asn tyr arg pro gly tyr phe thr gln thr ala gly pro trp his leu gly lys leu glu
901/301                                                              931/311
ttg gat ttc gac ttg tgt gaa ggc acc aca gtt gtt gtg gat gaa cat tgt gga aat cga
leu asp phe asp leu cys glu gly thr thr val val val asp glu his cys gly asn arg
961/321                                                              991/331
ggt cca tct ctc aga act aca aca gtc aca gga aag ata atc cat gaa tgg tgt tgc aga
gly pro ser leu arg thr thr thr val thr gly lys ile ile his glu trp cys cys arg
1021/341                                                             1051/351
tcc tgc acg tta ccc ccc tta cgc ttc aga gga gaa gac gga tgt tgg tat ggc atg gaa
ser cys thr leu pro pro leu arg phe arg gly glu asp gly cys trp tyr gly met glu
1081/361                                                             1111/371
atc aga cca gtt aag gag aag gag gag aac cta gtt agg tca atg gtc tct gca taa
ile arg pro val lys glu lys glu glu asn leu val arg ser met val ser ala
```

FIG.2

I.D.	IHA				MAC	O.D. during NS1 assay		
	D1	D2	D3	D4	ELISA	10 th	30 th	90 th
NOL 1	0	0	0	NR	NEG	0.24	0.16	0.13
NOL 2	10	40	10	NR	POS	0.13	0.10	0.09
NOT 1	0	0	0	NR	NEG	0.53	0.26	0.14
NOT 2	40	160	160	NR	POS	0.01	0.01	0.01
MONT 1	0	0	0	NR	NEG	1.02	0.67	0.44
MONT 2	320	40	20	NR	POS	0.03	0.02	0.02
BAIL 1	0	0	0	NR	NEG	1.20	0.83	0.64
BAIL 2	80	160	160	NR	POS	0.06	0.07	0.08
LEG 1	0	0	0	0	NEG	1.50	1.09	0.56
LEG 2	160	80	160	NR	POS	0.05	0.05	0.06
SGH 1	0	0	0	NR	NEG	0.76	0.54	0.26
SGH 2	80	640	160	NR	POS	0.03	0.02	0.03
DETT 1	0	0	0	NR	NEG	0.23	0.13	0.09
DETT 2	640	160	160	NR	POS	0.05	0.05	0.04
CON 1	0	0	0	0	NEG	0.23	0.10	0.10
CON 2	1280	1280	1280	1280	POS	0.12	0.07	0.03
BOLL 1	0	0	0	0	NEG	0.42	0.48	0.23
BOLL 2	1280	1280	1280	1280	POS	0.09	0.08	0.20
PORN 1	0	0	0	NR	NEG	0.54	0.54	0.44
PORN 2	1280	1280	1280	1280	POS	0.02	0.02	0.01
PAJ 1	0	0	0	0	NEG	1.71	1.27	0.73
PAJ 2	1280	1280	1280	1280	POS	0.07	0.07	0.06
PLAQ 1	10	20	20	NR	NEG	1.32	0.86	0.43
PLAQ 2	1280	1280	1280	1280	POS	0.28	0.18	0.18
GOH 1	10	40	20	NR	NEG	1.35	1.15	0.26
GOH 2	1280	1280	1280	1280	POS	0.06	0.09	0.14
BEAU 1	10	20	20	40	POS	1.01	0.76	0.53
BEAU 2	1280	1280	1280	1280	POS	0.07	0.08	0.07

Figure 3

	ELISA NS1+		ELISA NS1-		% POSITIVITY FOR NS1
	MAC IgM -	MAC IgM+	MAC IgM -	MAC IgM+	
D_0	2				-
D_1	13		3		81.2
D_2	8	1	6		64.3
D_3	10	4	1		93
D_4	4	6			100
D_5	1	6			100
D_6		4		1	80
D_7		1		1	-
D_8				1	-
D_9		1		2	-
D_{11} to D66				33	0

Figure 4

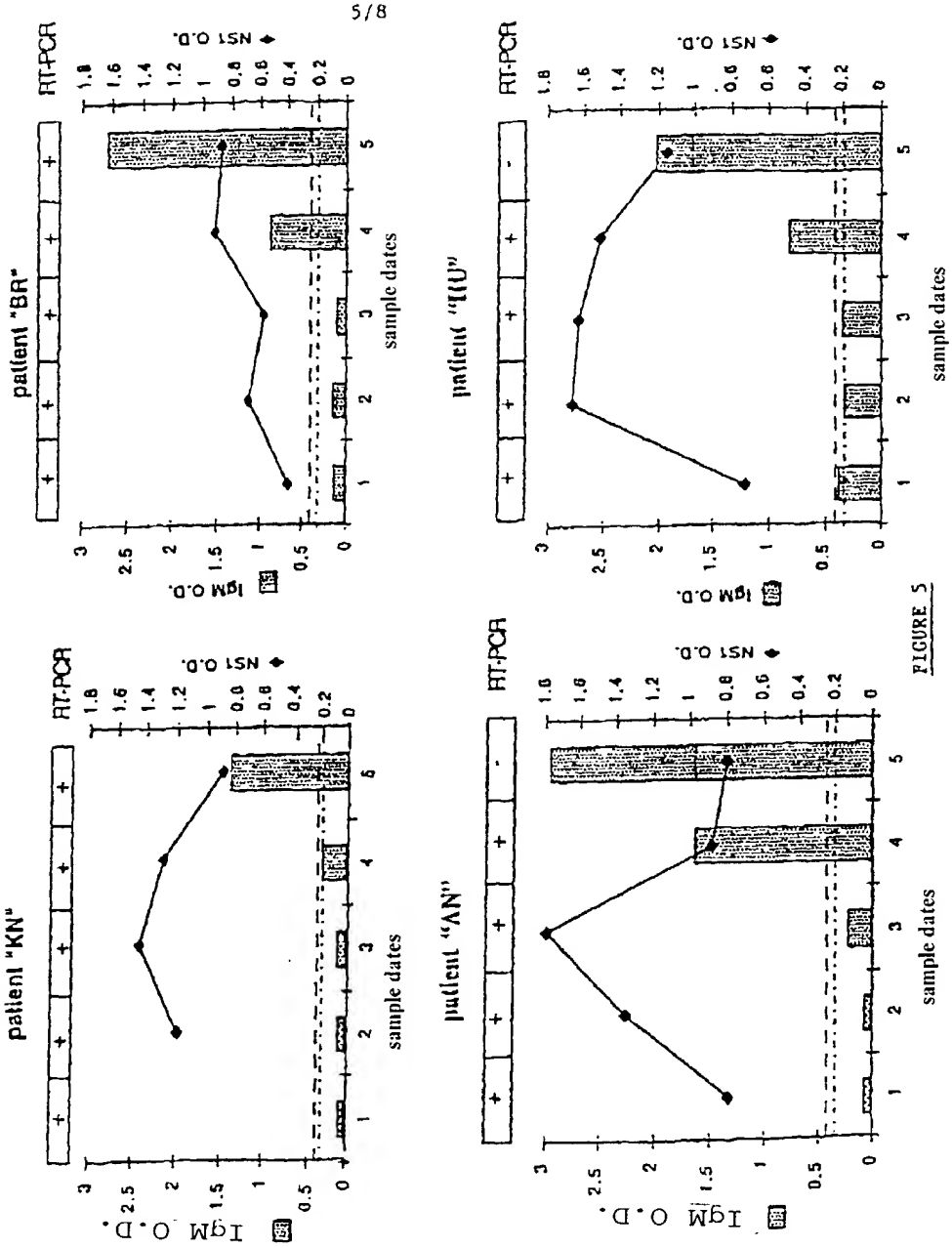
patient "KN"
patient "AN"
patient "BA"
patient "FU"
RT-PCR
IgM O.D.
NS1 O.D.
sample dates

FIGURE 5

	F22	G18
REACTIVITY IN ELISA (DEN1 NSI)		
purified soluble hexameric NS1	+	+
immunocaptured soluble hexameric NS1	+	+
REACTIVITY IN INDIRECT IMMUNOFLUORESCENCE		
DEN1 - infected cells	+	+
DEN2 - infected cells	-	-
DEN3 - infected cells	-	+
DEN4 - infected cells	-	-
ISOTYPE	IgG1 K	IgG1 K
AFFINITY CONSTANT	$2.7.10^{-9}$ M	3.10^{-11} M
EPITOPE	A	B

Figure 6

patient no.	DS	IgM	O.D. with monoclonals			O.D. with polyclonals		
			10th	30th	90th	10th	30th	90th
314	1	-	0.33	0.22	0.10	0.11	0.07	0
231	3	-	0.80	0.68	0.33	0.41	0.22	0.05
292	3	-	0.84	0.74	0.43	0.47	0.33	0.16
304	3	+	1.23	0.92	0.59	0.66	0.41	0.22
371	3	+	1.10	1.10	0.81	0.68	0,49	0
88	4	-	1.24	1.27	1.19	1.13	0.95	0.35
106	5	+	1.28	1.25	1.26	0.95	0.86	0.16
383	13	+	0.04	0.04	0.04	0.09	0.07	0.01
222	29	+	0.01	0.01	0.02	0.07	0.06	0
267	50	+	0.01	0.01	0	0.06	0.05	0.03

FIGURE 7

Serum No.	OD MAC IgM FJ	OD NS1 FJ	viral isolation
1	0.306	1.13	+
2	0.535	1.20	+
3	0.364	0.6	
4	0.578	0.65	
5	0.741	0.75	
6	0.968		
7	1.013		
8	1.101		
9	1.159		
10	1.278	0.61	
111	1.336		
12	1.448		
13	1.466		
14	1.501		
15	1.523	1.19	
16	1.587		
17	1.940		
18	2.109		

Figure 8

VOL TI -
Customer Number 22,852
Attorney Docket No. 03495.0213

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

EARLY DETECTION OF FLAVIVIRUSES USING THE NS1 GLYCOPROTEIN

the specification of which
☐ is attached and/or
☒ was filed on December 7, 2001 as United States Application Serial No. or
☒ PCT International Application No. PCT/FR00/01620 filed June 9, 2000.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
France	99/07290	June 9, 1999	☒ YES ☐ NO
France	99/07361	June 10, 1999	☒ YES ☐ NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., CUSTOMER NUMBER 22,852,** Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilley, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewis, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220;

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

100

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